Synthesis and Potent Antitumor Activities of Novel 1,3,5-*cis,cis*-Triaminocyclohexane *N*-Pyridyl Derivatives

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The iron chelator *N*,*N*′,*N*″-tris(2-pyridylmethyl)-*cis*,*cis*-1,3,5-triaminocyclohexane (tachpyr) was recently reported to display potent antitumor activity. The present study was focused on identifying an adequate bifunctional version of tachpyr as a lead compound for use in antibody-targeted iron depletion tumor therapy. Preparation of tachpyr derivatives having a side chain is reported, and their cytotoxic activity is evaluated in the HeLa cell line. The observed cytotoxity appears dependent on the functionalization site of the tachpyr employed for introducing the protein conjugation. Tachpyr derivatives **14** and **15** having a side chain introduced into the 5-position of the pyridyl ring display more potent cytotoxicity than tachpyr derivatives **7** and **8** having a side chain introduced onto one of the secondary amines. BOC-protected tachpyr derivative **14** exhibited the most potent cytotoxicity against this cancer cell line, which was reasonably comparable to the parent tachpyr. Tachpyr derivative **14** was further converted into bifunctional tachpyr **17** possessing a maleimide linker for conjugation with thiolated monoclonal antibodies (mAbs).

Introduction

The great promise of iron depletion as a cancer treatment strategy[1] has stimulated research on development of iron chelators as anticancer agents.[2] Among the iron chelators being evaluated or employed as antitumor agents are desferrioxmine (DFO),[3] Triapine,[4] and pyridoxal isonicotinoyl hydrazone (PIH) derivatives.[5] These iron chelators are known to cause cellular iron depletion and exhibit potent cytotoxic activities on numerous cancer cells including leukemia, neuroblastoma, hepatoma xenografts, and lymphoma.[3−5] Many studies to investigate mechanisms for the marked inhibitory activities of the iron chelators on cancer cells have been carried out. One mechanism may involve the inhibition of key iron-dependent enzymes. For example, DFO[3d] and Triapine[4b] have been shown to inhibit ribonucleotide reductase, an iron-dependent enzyme overexpressed in some tumor tissues.[1b,4a]

Our research has been focused on developing potent iron chelators for use in iron depletion tumor therapy. Recently, we reported that a novel iron chelator, *N*,*N*′,*N*″-tris(2-pyridylmethyl)-*cis*,*cis*-1,3,5-triaminocyclohexane (tachpyr, **3**),[6] exhibits antitumor effects on cultured cancer cells.[7] Iron complexes of tachpyr were nontoxic, suggesting that the capacity to bind metals is important to the cytotoxic activity of this chelator.[7a] The most striking finding was that tachpyr exhibited antitumor activity irrespective of activation of the p53 tumor suppressor gene.[7c] Tumors without the gene have been reported to display significantly diminished sensitivity to chemotherapeutic agents or angiogenesis inhibitors as compared to the tumors with the p53 gene.[8] Considering that ∼50% of human tumors are deficient in the p53 gene,[8d] tachpyr possesses great promise not only as a single antitumor agent but for use in combination with other chemotherapeutic agents.

A potential limitation of tachpyr as a clinically viable anticancer drug that requires consideration is a relatively short biological half-life. One approach to address this limitation and enhance selective targeting is to link tachpyr to a biomolecule such as a monoclonal antibody (mAb). To test this approach, we proposed to design and synthesize a bifunctional version of tachpyr possessing a linker for conjugation with mAbs. We were particularly interested in preparation of a bifunctional tachpyr possessing an electrophilic maleimide group, which would readily undergo Michael addition with a nucleophilic thiol group. Thus, a bifunctional tachpyr might be readily conjugated to a selection of thiolated mAbs using established conjugation chemistry to afford a variety of antitumor conjugates that will possess a more favorable biological half-life and allow for the selective targeted delivery of tachpyr cytotoxicity.

As an ongoing effort to develop an adequate bifunctional version of tachpyr without sacrificing its cytotoxicity, we have designed a novel series of tachpyr analogues substituted with different side chains and investigated their ability to act as cytotoxic agents. On the basis of the observed cytotoxicity of the tachpyr analogues described herein, we have proceeded to the design and preparation of the bifunctional tachpyr **17** possessing a maleimide moiety. Herein, we now report the synthesis of the tachpyr analogues and the bifunctional tachpyr **17**. The cytotoxic activity of the tachpyr analogues is measured in Hela cells using a (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay and is compared to that of the parent compound, tachpyr.

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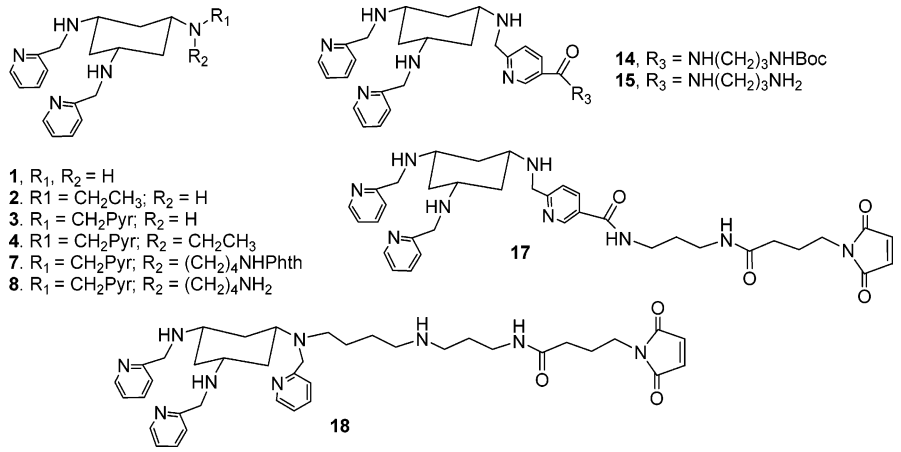
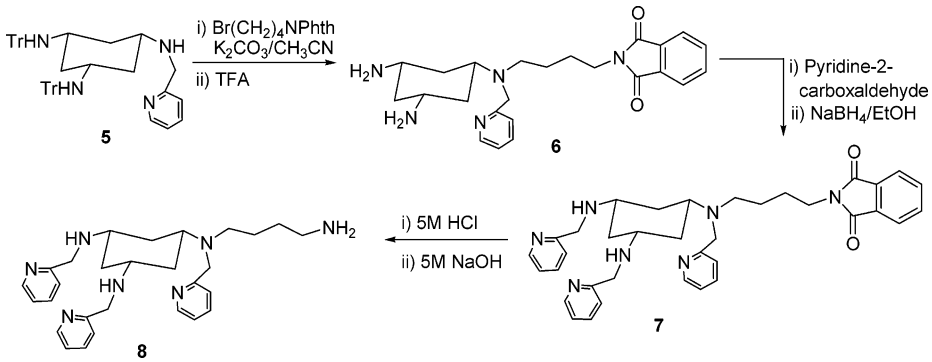
Scheme 1



Scheme 2



Result and Discussions

Tachpyr analogues **7**, **8**, **14**, and **15** possessing different side chains designed for the present study are shown in Scheme 1. Two different types of structural modifications to the tachpyr framework are sought to investigate the effect of a side chain on the cytotoxic activity of tachpyr. While analogues **7** and **8** possess a side chain introduced onto one of the secondary amines of tachpyr, analogues **14** and **15** possess a side chain introduced into the 5-position of a pyridyl group in tachpyr. Previously, we reported a convenient synthesis of tach *N*-pyridyl derivatives via selective partial protection of the amines in *cis*,*cis*-1,3,5-triaminocyclohexane (tach) employing the bulky trityl (Tr) group.[9] The synthetic techniques reported therein have been directly applied to the preparation of tachpyr analogues for the present structure−activity relationship study. Tachpyr analogues **7** and **8** were synthesized as previously published (Scheme 2).[9]

The synthetic approach to tachpyr analogues **14** and **15** is shown in Scheme 3. Thus, commercially available diester **9** was selectively reduced to **10** via a modification of a previously published procedure.[10] The hydroxyester **10** was reacted with 1,3-diaminopropane to afford the intermediate 2-aminoethylamide **11**, which was subsequently reacted with BOC−ON to afford carbamate **12**. BOC-protected **12** was oxidized (MnO_2) to provide aldehyde **13** in 92% yield. Reaction of **13** with bis(pyridylmethyl)tach **1**[9] and subsequent reduction with $NaBH_4$ provided **14**. The BOC group in **14** was removed by treating with HCl to provide amine **15**.

The cytotoxicity of tachpyr analogues **7**, **8**, **14**, and **15** was evaluated using the HeLa cell line as described previously (Figure 1).[11] The data in Figure 1 show that

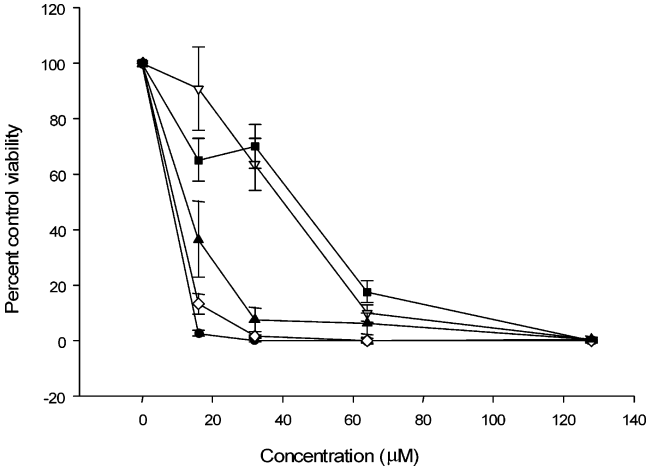
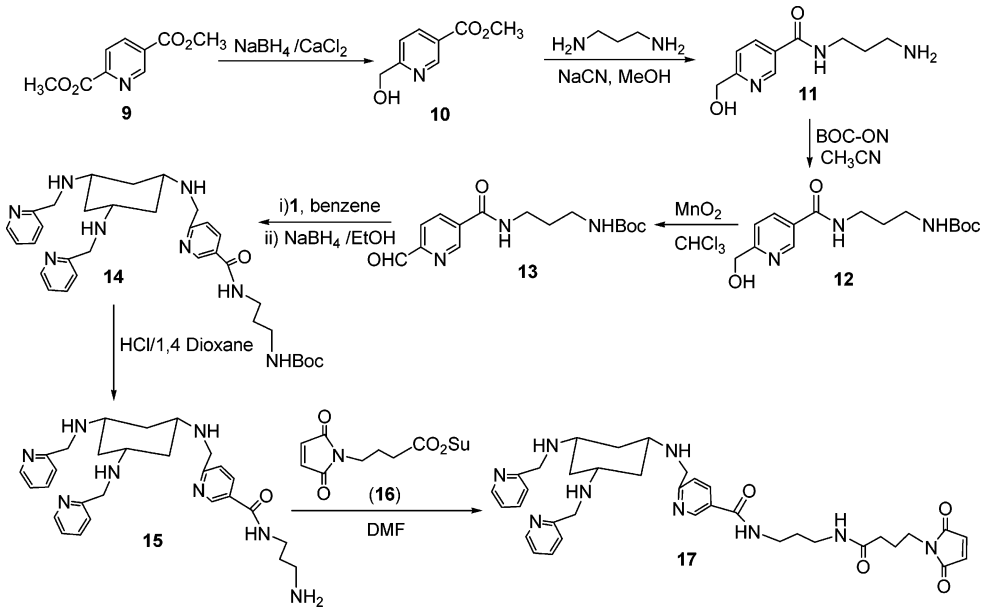


Figure 1. Effects of chelators **7** (▼), **8** (■), **14** (◇), **15** (▲), and tachpyr (●) on cell viability. Cells were incubated for 72 h with chelators at various concentrations, and viability was assessed as described in Materials and Methods. Each point represents the mean and standard error of octuplicate cultures.

introduction of a side chain into the 5-position of the pyridyl ring in tachpyr (**14**) is well tolerated and resulted in minimal decrease in cytotoxicity as compared with tachpyr. However, analogues **7** and **8**, N-functionalized at a secondary amine, display reduced cytotoxicity as compared to C-functionalized tachpyr **14** and **15** over the entire concentration range evaluated (Figure 1). Nonetheless, all of the tachpyr analogues reported herein are more cytotoxic agents than the previously described tachpyr analogues **1** and **2** (Figure 2).[9] We had

Scheme 3



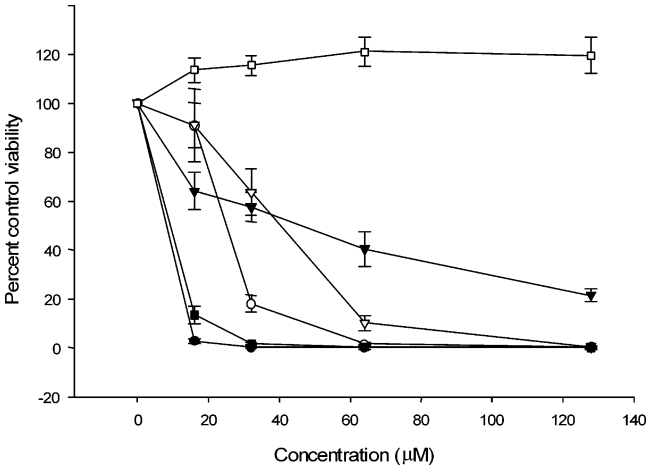


Figure 2. Effects of chelators **1** (▼), **2** (□), **4** (○), **7** (▽), **14** (■), and tachpyr (●) on cell viability. Cells were incubated for 72 h with chelators at various concentrations, and viability was assessed as described in Materials and Methods. Each point represents the mean and standard error of octuplicate cultures.

previously reported that tachpyr analogues **1** and **2** exhibited significantly decreased cytotoxicity as compared to tachpyr **3**.[9] We had concluded from our prior study that replacement of a pyridyl group in tachpyr **3** by either hydrogen (**1**) or a propyl group (**2**) resulted in a significant decrease in cytotoxicity and that all three pyridyl groups of tachpyr should be retained in structural modifications of **3**.[9] The present study further demonstrates that the presence of the six coordinating groups, that is, three secondary amines and three pyridylamines is required for maintaining the cytotoxic activity of tachpyr analogues. It is interesting to note that removal of the protecting groups (BOC or Phth) from **7** and **14**, respectively, caused a slight decrease in cytotoxic activity. At this point, it is not clear whether the distant primary naked amine might participate in iron binding and somehow disturb the geometry and stability of the six-coordinating binding sphere of tachpyr, thereby leading to reduced cytotoxicity. Among all of tachpyr derivatives, the BOC-protected tachpyr derivative **14** displayed the highest activity against HeLa cells and was reasonably comparable to **3**. Reactions were carried out to convert **14** into bifunctional tachpyr **17** possessing a maleimide linker for conjugation to thiolated antibody (Scheme 3). Thus, reaction of **15** with the active ester reagent **16**[12] followed by silica gel flash column chromatorgraphic purification provided **17** in 66% yield.

Although the antitumor efficacy of antibody conjugates based on the model compound **17** remains to be determined, previous studies have demonstrated that antibody conjugation can improve drug performance in several ways, including a reduction in systemic toxicity, increased potency, and prolonged half-life.[13] Conjugated mAbs have also shown a dramatic increase in antitumor activity when used in combination therapy.[14] We are currently investigating biological activity of antitumor conjugates of C-functionalized tachpyr **17**, along with N-functionalized tachpyr **18**[9] prepared by reaction of **8** and **16** as previously described.

Conclusion

We have prepared novel tachpyr analogues and measured their cytotoxic activities on Hela cells. The present study shows that choice of functionalization site in tachpyr has meaningful impact on the cytotoxic activity of tachpyr. The result of the cytotoxic assay demonstrates that while a pyridyl ring moiety could be substituted with a side chain without significant decrease in the cytotoxic activity of tachpyr, substitution of one of three secondary amines with an analogous side chain could result in decreased activity. Tachpyr derivatives described herein will serve as the basis for our continuing structure−activity relationship study of these tach-derived iron chelators as antitumor drugs. Future plans include an examination of impact on the position of the pyridyl ring for functionalization on the cytotoxicity. Studies are also ongoing with tach derivatives having substituents other than pyridyl groups.

In sum, we have successfully prepared a bifunctional tachpyr ligand **17** that possesses great promise as a cancer therapeutic. Bifunctional ligand **17** might be linked to a number of monoclonal antibodies targeting

to various types of tumor cells to generate the first series of antitumor conjugates for use in targeted iron depletion tumor therapy. Studies pertaining to the development of conjugation chemistry, tumor cell targeting, and efficacy are ongoing and will be reported in due course.

Experimental Section

General. ^{1}H, ^{13}C, and APT NMR spectra were obtained at 300 MHz in $CDCl_3$ solution. Fast atom bombardment mass spectra (FAB-MS) were obtained in the positive ion detection mode. Elemental microanalysis was performed by Galbraith Laboratories, Knoxville, TN.

6-Hydroxymethyl-nicotinic Acid Methyl Ester 10. $NaBH_4$ (149 mg, 3.95 mmol) was added to a slurry of pyridine 2,5-dicarboxylate (308 mg, 1.58 mmol) and $CaCl_2$ (693 mg, 6.25 mmol) in THF (3.3 mL) and EtOH (6.7 mL) at 0 °C in portions. The resulting mixture was stirred at 0 °C for 7 h, poured into ice−water (5 mL), and extracted with $CHCl_3$ (3 × 20 mL). The combined organic layers were dried ($MgSO_4$) and filtered, and the filtrate was concentrated in vacuo to provide crude **10** (264 mg, 92%) as a colorless solid. The ^{1}H NMR and ^{13}C NMR spectra of the material thereby obtained were essentially identical to data reported previously for authentic **10**.[10] ^{1}H NMR ($CDCl_3$) δ 3.89 (s, 3 H), 4.42 (s, 1 H), 4.78 (s, 2 H), 7.38 (d, J = 7.9 Hz, 1 H), 8.22 (dd, J = 2.3 Hz, 1 H), 9.05 (s, 1 H); ^{13}C NMR ($CDCl_3$) δ 52.3 (q), 64.3 (t), 120.0 (d), 124.6 (s), 137.8 (d), 149.8 (d), 164.1 (s), 165.5 (s).

***N*-(3-Amino-propyl)-6-hydroxymethyl-nicotinamide 11.** A mixture of **10** (6.68 g, 40 mmol), 1,3-diaminopropane (40 mL, 472 mmol), and NaCN (252 mg, 5.05 mmol) in MeOH (500 mL) was refluxed for 24 h. The resulting mixture was concentrated in vacuo, and EtOH (100 mL) was added into the residue. The resulting mixture was stirred for 5 min and put into the freezer for 1 h. The mixture was filtered, and the filtrate was concentrated in vacuo. The residue was dissolved into $CHCl_3$ (100 mL), and the mixture was stirred for 4 h at room temperature and filtered. The filtrate was dried ($MgSO_4$), refiltered, and concentrated in vacuo to afford crude **11** (3.90 g, 43%): ^{1}H NMR ($CDCl_3$) δ 0.98−1.02 (m, 2 H), 1.90−1.98 (m, 2 H), 2.64−2.70 (m, 2 H), 3.95 (s, 2 H), 4.17 (s, 4 H), 6.84 (d, J = 7.4 Hz, 1 H), 7.42 (dd, J = 3.2 Hz, 1 H), 8.09 (s, 1 H); ^{13}C NMR ($CDCl_3$) δ 33.2 (t), 38.5 (t), 39.9 (t), 65.4 (t), 121.5 (d), 130.2 (s), 137.5 (d), 148.7 (d), 165.7 (s), 167.9 (s). The crude product was used directly in the next step.

{3-[(6-Hydroxymethyl-pyridine-3-carbonyl)-amino]-propyl}-carbamic Acid *tert*-Butyl Ester 12. A solution of BOC−ON (4.47 g, 18.2 mmol) in CH_3CN (40 mL) was added dropwise to a slurry of **11** (3.5 g, 16.5 mmol) in CH_3CN (60 mL) over 1 h. The resulting mixture was stirred for 7 h at room temperature. The solvent was evaporated, and the residue was dissolved into ether (100 mL). The mixture was washed with 20% NaOH (50 mL) and H_2O (2 × 50 mL). The ether layer was dried ($MgSO_4$), filtered, and evaporated. The residue was purified on silica gel chromatography eluted with 8% MeOH in CH_2Cl_2 to provide pure **12** (4.45 g, 87%) as a colorless solid: ^{1}H NMR ($CDCl_3$) δ 1.44 (s, 9 H), 1.69−1.74 (m, 2 H), 3.26 (q, 2 H), 3.50 (q, 2 H), 4.80 (s, 2 H), 4.96 (t, J = 7.2 Hz, 1 H), 7.34 (d, J = 6.9 Hz, 1 H), 7.77 (s, 1 H), 8.16 (d, J = 7.6 Hz, 1 H), 9.01 (s, 1 H); ^{13}C NMR ($CDCl_3$) δ 28.3 (q), 29.9 (t), 35.9 (t), 36.9 (t), 64.2 (t), 79.8 (s), 120.1 (d), 129.0 (s), 135.7 (d), 147.3 (d), 157.2 (s), 162.0 (s), 165.3 (s). HRMS (positive ion FAB) Calcd for $C_{15}H_{23}N_3O_1$: $[M + H]^+$ m/z 310.1778. Found: $[M + H]^+$ m/z 310.1767. Anal. ($C_{15}H_{23}N_3O_4 \cdot 0.5H_2O$) C, H.

{3-[(6-Formyl-pyridine-3-carbonyl)-amino]-propyl}-carbamic Acid *tert*-Butyl Ester 13. A mixture of **12** (1.78 g, 5.74 mmol) and MnO_2 (4.69 g, 54 mmol) in $CHCl_3$ (20 mL) was refluxed for 5 h. The resulting mixture was filtered, and the filtrate was concentrated in vacuo. The residue was purified using silica gel column chromatography eluted with 5% MeOH in CH_2Cl_2 to provide pure **13** (1.69 g, 96%) as a colorless solid: ^{1}H NMR ($CDCl_3$) δ 1.46 (s, 9 H), 1.70−1.78 (m, 2 H), 3.26−3.32 (m, 2 H), 3.52−3.58 (m, 2 H), 4.84 (t, J = 6.8 Hz, 2 H), 8.04 (d, J = 8.1 Hz, 1 H), 8.37 (dd, J = 2.1 Hz, 1 H), 9.27 (s, 1 H), 10.1 (s, 1 H); ^{13}C NMR ($CDCl_3$) δ 28.5 (q), 29.9 (t), 36.8 (t), 37.3 (t), 79.7 (s), 121.5 (d), 133.6 (s), 136.3 (d), 149.2 (d), 154.0 (s), 157.4 (s), 164.9 (s), 192.8 (s). HRMS (positive ion FAB) Calcd for $C_{15}H_{21}N_3O_4$: $[M + H]^+$ m/z 308.1622. Found: $[M + H]^+$ m/z 308.1610. Anal. ($C_{15}H_{21}N_3O_4 \cdot 0.5H_2O$) C, H.

(3-{[6-({3,5-Bis-[(pyridin-2-ylmethyl)-amino]-cyclohexylamino}-methyl)-pyridine-3-carbonyl]-amino}-propyl)-carbamic Acid *tert*-Butyl Ester 14. To a solution of **1** (350 mg, 1.13 mmol) in benzene (25 mL) was added pyridinecarboxaldehyde (347 mg, 1.13 mmol). The resulting mixture was refluxed using a Dean−Stark trap for 18 h. The reaction mixture was cooled to the room temperature and evaporated to dryness to provide pure imine as determined by NMR. The obtained imine was dissolved in EtOH (10 mL) and reacted with $NaBH_4$ (43 mg, 1.13 mmol). The resulting mixture was stirred at room temperature for 24 h and filtered, and the filtrate was concentrated in vacuo. The residue was dissolved in CH_2Cl_2 (15 mL), filtered again and dried ($MgSO_4$), and the filtrate was concentrated in vacuo to provide crude **14**. The crude product was suitable for next step or could be purified via column chromatography on silica gel eluting with 0.8 mL Et_3N/15 mL MeOH/100 mL CH_2Cl_2. Pure **14** (771 mg, 93%) was thereby obtained as a colorless oil: ^{1}H NMR ($CDCl_3$) δ 1.01 (dd, J = 7.4 Hz, 4 H), 1.36 (s, 9 H), 1.54−1.65 (m, 2 H), 1.80 (s, 2 H), 2.17−2.21 (m, 3 H), 2.48−2.56 (m, 3 H), 3,14−3.18 (m, 2 H), 3.35−3.45 (m, 2 H), 3.87 (s, 4 H), 3.92 (s, 2H), 5.26 (t, J = 2.6 Hz, 1 H), 7.07 (dd, J = 5.4 Hz, 2 H), 7.21 (d, J = 7.6 Hz, 2 H), 7.31 (d, J = 7.6 Hz, 1 H), 7.56 (dt, J = 10 and 2.1 Hz, 2 H), 7.81 (s, 1 H), 8.06 (dd, J = 2.4 Hz, 1 H), 8.46 (d, J = 5.2 Hz, 2 H), 8.96 (s, 1 H); ^{13}C NMR ($CDCl_3$) δ 27.8 (q), 29.3 (t), 35.7 (t), 36.4 (t), 39.6 (2C, t), 51.5 (t), 51.7 (t), 53.0 (2C, d), 121.1 (d), 121.3 (d), 121.7 (d), 127.9 (s), 134.8 (d), 135.8 (d), 147.5 (d), 148.5 (d), 156.5 (s), 159.0 (s), 162.1 (s), 165.1 (s). HRMS (positive ion FAB) Calcd for $C_{33}H_{46}N_8O_3$: $[M + Cs]^+$ m/z 735.2729. Found: $[M + H]^+$ m/z 735.2747.

***N*-(3-Amino-propyl)-6-({3,5-bis-[(pyridin-2-ylmethyl)-amino]-cyclohexylamino}-methyl)-nicotinamide 15.** Four molar HCl in dioxane (5 mL) was slowly added to **14** (360 mg, 0.6 mmol) at 0 °C. The resulting mixture was warmed to room temperature and stirred for 18 h. Ether (40 mL) was added into the mixture, and the mixture was held in the freezer for 3 h. The tan solid was filtered and dissolved in H_2O (10 mL). The aqueous solution was adjusted to pH 7 with 5 M NaOH, washed with $CHCl_3$ (10 mL), and adjusted to pH 13. The aqueous solution was evaporated, and the residue was treated with $CHCl_3$ (3 × 50 mL). The combined organic layers were dried ($MgSO_4$) and filtered, and the filtrate was concentrated in vacuo to provide pure **15** (357 mg, 94%). ^{1}H NMR ($CDCl_3$) δ 0.88 (dd, J = 7.4 Hz, 4 H), 1.55−1.85 (m, 5 H), 2.08−2.18 (m, 3 H), 2.36−2.50 (m, 3 H), 2.72 (t, J = 5.8 Hz, 2 H), 3,38−3.45 (m, 2 H), 3.75 (s, 4 H), 3.81 (s, 2H), 7.07 (dd, J = 6.4 Hz, 2 H), 7.13 (d, J = 7.6 Hz, 2 H), 7.26 (d, J = 7.6 Hz, 1 H), 7.46 (dt, J = 8.7 and 2.1 Hz, 2 H), 7.93 (dd, J = 2.5 Hz, 1 H), 8.36 (d, J = 3.7 Hz, 2 H), 8.53 (s, 1 H), 8.80 (s, 1 H); ^{13}C NMR ($CDCl_3$) δ 30.6 (t), 39.1 (t), 39.9 (2C, t), 40.4 (t), 51.8 (t), 52.0 (t), 53.3 (d), 121.4 (d), 121.5 (d), 121.9 (d), 128.3 (s), 135.1 (d), 136.1 (d), 147.4 (d), 148.7 (d), 159.3 (s), 162.3 (s), 165.1 (s). HRMS (positive ion FAB) Calcd for $C_{28}H_{38}N_8O$: $[M + Cs]^+$ m/z 635.2223. Found: $[M + Cs]^+$ m/z 635.2198.

6-({3,5-Bis-[(pyridin-2-ylmethyl)-amino]-cyclohexylamino}-methyl)-*N*-{3-[4-(2,5-dioxo-2,5-dihydro-pyrrol-1-yl)-butyrylamino]-propyl}-nicotinamide 17. To a solution of **15** (120 mg, 0.24 mmol) in anhydrous DMF (4 mL) was added active ester **16** (67 mg, 0.24 mmol). The resulting mixture was stirred for 18 h at room temperature, and solvent was removed via high-vacuum rotary evaporation at room temperature. The residue was purified via neutral alumina flash chromatograpy eluting with 8% methanol in CH_2Cl_2 to provide **17** (105 mg, 66%) as a colorless oil: ^{1}H NMR ($CDCl_3$) δ 1.18−1.34 (m, 4 H), 1.60−1.85 (m, 3 H), 1.90−1.99 (m, 3 H), 2.17−2.34 (m, 3 H), 2.53−2.82 (m, 5 H), 3.36 (q, 2 H), 3.50 (q, 2 H), 3.58 (t, 2 H), 3.98 (s, 4 H), 4.01 (s, 2 H), 6.52 (t, J = 8.0 Hz, 1 H), 6.70

(s, 2 H), 7.15 (ddd, $J = 11.2$, 6, and 2.4 Hz, 2 H), 7.32 (d, 2 H), 7.38 (d, 1 H), 7.64 (dt, $J = 9.2$ and 2 H), 7.87 (t, $J = 8.0$ Hz, 1 H), 8.13 (dd, $J = 8$ and 2.4 Hz, 1 H), 8.51 (d, $J = 6.0$ Hz, 2 H), 9.04 (d, $J = 1.6$ Hz, 1 H); ^{13}C NMR ($CDCl_3$) δ 24.7 (t), 29.3 (t), 33.5 (t), 35.9 (t), 36.0 (t), 37.13 (t), 37.93 (t), 51.6 (t), 51.77 (t), 51.79 (t), 53.40 (d), 53.46 (d), 122.1 (d), 122.4 (d), 122.7 (d), 128.8 (d), 134.2 (d), 135.7 (d), 136.7 (d), 148.2 (d), 149.2 (d), 157.8 (s), 161.4 (s), 165.6 (s), 170.9 (s), 172.9 (s); MS (positive ion FAB) Calcd for $C_{36}H_{45}N_9O_4$: $[M + H]^+$ *m/z* 668.36. Found: $[M + H]^+$ *m/z* 668.38.

Cytotoxicity Assay. Hela cells were obtained from the American Type Culture Collection and grown in a humidified 5% CO_2 atmosphere at 37 °C in DME medium (Gibco BRL) supplemented with 10% fetal bovine serum and penicillin/streptomycin.

A total of 2×10^3 cells were plated in 96 well tissue culture dishes and allowed to attach overnight before test compounds were added. Six replicate cultures were used for each point. After 72 h, viability was assessed using an MTT assay, in which (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide is added to the medium and the formation of a reduced product is assayed by measuring the optical density at 560/650 nm after 3 h. Color formation is proportional to viable cell number.[11]

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